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Plasmodium berghei berghei: Irradiated Sporozoites of the ANKA Strain as Immunizing Antigens in Mice[1]

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INDEX DESCRIPTORS: Sporozoite; *Plasmodium berghei berghei*; Parasitic protozoa; ANKA strain; Immunizaion; Malaria; Mice; Vaccine; X-ray; *Anopheles stephensi*; Mosquito vector; Antigen.

INTRODUCTION

Nussenzweig *et al.* (1967) were the first to report sterile immunity against rodent malaria following vaccination with irradiated sporozoites. Previous attempts to artificially induce immunity using the sporozoite as an immunogen date back to the first decade of this century (Sergent and Sergent 1910). However, these workers, as well as their successors, achieved limited success in that they were able only to stimulate a form of immunity which became known as premunition (Sergent *et al.* 1924).

To date, the landmark experiments with *Plasmodium berghei* in mice reported by Nussenzweig and her co-workers have not been confirmed by an independent laboratory, although results using different host systems have appeared (Clyde *et al.* 1973; Rieckmann *et al.* 1974; Ward and Hayes 1972; Collins and Contacos 1972).

All the attempts to immunize with sporozoites cited above have not been uniformly successful. Nonetheless, the success which has been achieved clearly points to the importance and potential of the approach while emphasizing the need for a better understanding of the underlying biological principles which are operative. These prin-

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ciples can only be understood following experimentation with animal models, and the rodent plasmodia appear to offer the best system for such studies.

The present study reports the details of an experimental system for immunizing NIH/Nmri mice against infection with the ANKA strain of *Plasmodium berghei* using irradiated sporozoites as the immunogen. The initial experiments were designed to: (1) confirm the work of Nussenzweig *et al.*, since conflicting results have appeared (Ward and Hayes 1972; Collins and Contacos 1972); (2) validate the new mosquito–rodent–parasite combination used in this laboratory as a suitable model for sporozoite immunization studies; (3) establish standards for the model against which subsequent results can be compared; and (4) examine the alternative hypothesis advanced by Alger *et al.* (1972) regarding the specificity of the immune response to sporozoites.

Materials and Methods

The vector, host, parasite combination used in these studies was *Anopheles stephensi*, NIH/Nmri albino mice, and the ANKA strain of *Plasmodium berghei berghei*. Female mice were used to minimize aggressive behavior during the lengthy immunization schedule. Mosquitoes were infected with *P. berghei* by allowing them to feed on infected mice (Beaudoin *et al.* 1974b). The experimental designs for the three experiments are outlined in Fig. 1. The infected mosquitoes in Experiments 1 and 2 were irradiated at 18 days postinfection with approximately 8000 R delivered over a 10-min period using a GE 300 KVP X-ray machine. Following irradiation of infected mosquitoes, thoraxes containing the salivary glands were separated from abdomens, placed in Medium 199 (M199), comminuted by grinding, and filtered through a fine mesh screen to re-

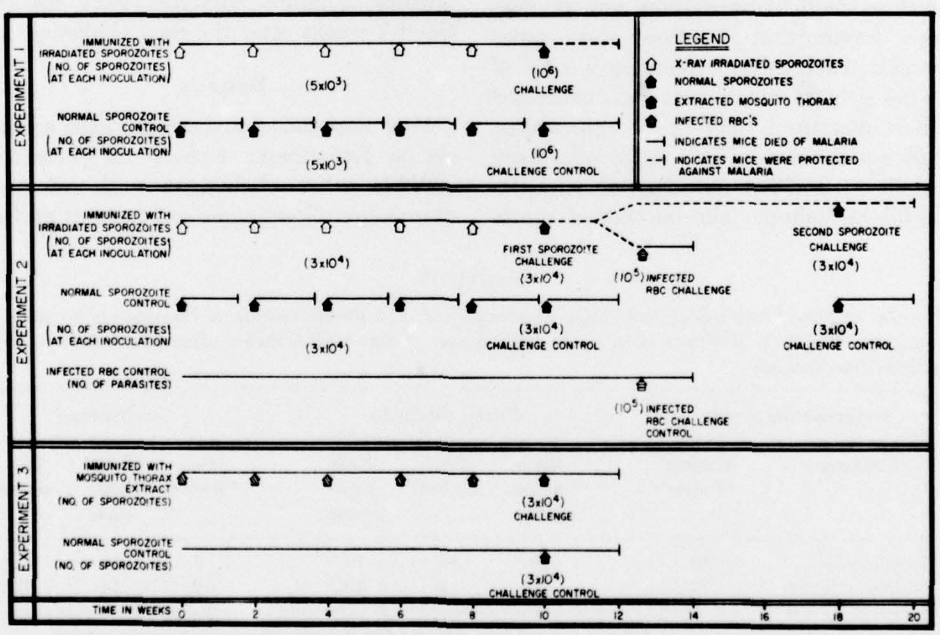

Fig. 1. Experimental design for *Plasmodium berghei berghei*: Irradiated sporozoites of the ANKA strain as immunizing antigens in mice.

TABLE I

Number of Mice Protected against Challenge with 10^5 Sporozoites of *Plasmodium berghei* after Immunization with Irradiated Sporozoites of the ANKA strain[a]

Experimental group		Number of mice	
Treatment	Number	Challenged	Protected[b]
Immunized	20	20	20
Nonimmunized	20	20	0

[a] Immunization schedule consisted of five doses of 5×10^4 irradiated sporozoites inoculated i.v. at biweekly intervals.
[b] Animals were considered protected if they did not become infected after challenge.

move large particles. The filtrate was centrifuged at 200*g* for 3 min. Sporozoites were recovered in the supernatant fluid, washed in M199, centrifuged at 16,000*g* for 25 min at 4 C. The supernatant fluid was discarded and the sporozoite button reconstituted in 1 ml of M199, and a sample counted in a standard hemocytometer. The concentration of sporozoites was then adjusted to the desired number for one dose in a 0.1 ml vol. This was the volume administered into the tail vein of each mouse at each immunization and at challenge. Immunizing injections were given biweekly, each mouse receiving a total of five doses, after which each was challenged with a measured number of sporozoites. Challenge doses were likewise standardized to deliver specified numbers of parasites in a 0.1 ml volume. The number of sporozoites used both to immunize and challenge is given in Fig. 1 and in the appropriate table.

In Experiment 2, standard challenge doses of parasites of the red cell cycle were prepared after counting red blood cells in a hemocytometer, determining the percentage of parasitemia, and diluting the sample with M199 to achieve the desired inoculum.

Immunizing doses of mosquito antigens were prepared by grinding the thoraxes of uninfected mosquitoes in M199 and filtering as above. One-tenth milliliter of the filtrate was inoculated into the tail vein of each mouse undergoing immunization in Experiment 3.

To determine the efficacy of immunization, blood films were prepared from each mouse and searched for parasites at 6 days after challenge and at appropriate intervals thereafter. Blood was subinoculated into uninfected recipients from immune survivors 2 weeks after challenge to ensure that they did not harbor subpatent parasitemias. Immune survivors were usually killed 3 weeks after the final challenge.

Results

Mice immunized according to the schedule in Experiment 1 were all protected (20/20) when challenged with infective sporozoites. Nonimmunized controls all be-

TABLE II

Number of Mice Protected against Initial Challenge with 3×10^4 Sporozoites of *Plasmodium berghei* and Rechallenge with 3×10^4 Sporozoites or 10^5 RBC Stages of the ANKA Strain after Immunization with Irradiated Sporozoites[a]

Experimental group		Initial challenge		Rechallenge[b]			
Treatment	Number of mice	With sporozoites	Protected	With sporozoites	Protected	With RBC stage	Protected
Immunized	20	20	20	10	9	10	0
Nonimmunized	20	20	0	10	0	10	0

[a] Five immunizing doses of 3×10^4 sporozoites were administered during the immunization period.
[b] Twenty mice surviving the initial challenge were divided into equal groups of 10; one was rechallenged with sporozoites while the other was rechallenged with RBC stages.

came infected and none (0/20) survived (Table I). Attempts to detect subpatent infections by subinoculation of blood from surviving challenged mice into uninfected recipients failed to produce any infection in the recipient animals.

At the time of each immunization, but prior to irradiation, nonirradiated samples of sporozoites were set aside for inoculation into five normal mice to assure that sporozoites were viable before irradiation. These mice always became infected. The group of immune mice rechallenged 8 weeks after their first sporozoite challenge, as outlined in Experiment 2, remained protected (10/10) against the second sporozoite challenge (Table II). The group rechallenged with blood stages 2 weeks after the initial sporozoite challenge was completely susceptible or unprotected (0/10) against this form of challenge. Nonimmunized challenge controls for both blood and sporozoites all became infected and died.

With a single exception, mice immunized with M199 extracted uninfected mosquito thorax antigens (Experiment 3) produced parasitemias in all animals following their challenge with sporozoites (Table III). As expected, all of the nonimmunized animals became infected and died.

TABLE III

Number of Mice Protected against Challenge with 3×10^4 Sporozoites of *Plasmodium berghei* after Immunization with a Saline Extract of the Thoraxes of Uninfected *Anopheles stephensi*[a]

Experimental group		Number of mice	
Treatment	Number	Challenged	Noninfected
Immunized	10	10	1
Nonimmunized	10	10	0

[a] Saline extract consisted of M199 washings of macerated tissue excised from the mosquito.

Discussion

Our experience based upon results from these experiments suggests that it is possible to immunize mice successfully against sporozoite challenge with the ANKA strain of *P. berghei* and that the immunity is specific for the preerythrocytic phase of the infection.

These results agree with the data reported by Nussenzweig *et al.* (1969) from immunization experiments using mice and the NK65 strain of *P. berghei* transmitted by *Anopheles stephensi*, and confirm their description of immunization in the rodent model; the results also demonstrate the comparability of the two systems. There can be little question now that sporozoites effectively serve as a potent immunogen and confer a significant degree of protection against sporozoite challenge. Furthermore, use of this form of the parasite offers the distinct advantage that the immunity produced is a functionally sterile one. Circulating parasites, responsible for disease and transmission to mosquitoes, are never observed nor can the infection be transferred to nonimmune individuals by subinoculation of blood, although exoerythrocytic parasites may persist within liver cells for an undetermined period of time (Vanderberg *et al.* 1968).

This type of immunity can be contrasted to the immunity observed when parasites of the red blood cell cycle are used as the antigenic forms. Here, the immunity appears to be definitely of the nonsterile type, or classic premunition (Sergent *et al.* 1924; Taliaferro and Taliaferro 1929; Wellde *et al.* 1972; and many others), although Mitchell *et al.* (1974) have claimed sterile immunity in a single monkey immunized with merozoites of the erythrocytic cycle of *P. knowlesi*. The important characteristic of premunition is persistence of circulating parasites in the blood of the resistant host.

Beaudoin and Applegate (1972) have shown that in premunition the remaining subpatent infections persist in the host for long periods of time and that such hosts may act as reservoirs perpetuating introduced challenge strains indefinitely. Individuals with this type of immunity may be capable of infecting mosquitoes (Apple-

gate *et al.* 1971), thereby transmitting a parasite population with an exceedingly variable genetic composition. In addition, several investigators (Cox 1959; Brown and Brown 1965; and others) have reported that parasites of the red blood cell cycle show a strong tendency to form *de novo* antigenic variants which may also complicate attempts to immunize with these forms. This phenomenon has not yet been observed either in animals or humans immunized with sporozoites.

Irradiated sporozoites have also been used to immunize man (Clyde *et al.* 1973; Rieckmann *et al.* 1974). Human volunteers were protected against challenge with both homologous and heterologous strains of *Plasmodium falciparum* using a minimum of six inoculations of irradiated sporozoites. However, Rieckmann (1974) has reported that fewer than six immunizing exposures failed to elicit protection. Thus, the failure to produce complete protective immunity in nonhuman primates reported by Ward and Hayes (1972) and Collins and Contacos (1972) may be simply a failure to provide sufficient antigen to reach the threshold levels. This point is further emphasized by the results of Collins and Contacos in that the amount of antigenic stimulus provided to the experimental animals was approximately twice that provided in the studies reported by Ward and Hayes, and the challenge in this study produced a measurably delayed onset and reduced parasitemia as compared to the Ward and Hayes study.

On the other hand, our data do not support the hypothesis advanced by Alger *et al.* (1972) that mosquito antigens adsorbed to the surface of the sporozoite may be responsible, at least in part, for the protection elicited. In our experiments, the specificity of the immunity was shown to be directly against sporozoites, since attempts to immunize mice with mosquito thorax antigens failed to produce a significant level of protection. This discrepancy in the two sets of results may be due to differences in the amounts of mosquito antigen and the routes of immunization and challenge used in the two studies. The mosquito antigens in our studies (Experiment 3) were prepared by a method similar to that used for preparing sporozoites. This method undoubtedly results in a lowered concentration of mosquito antigens than was used by Alger and her co-workers. Secondly, it is important to note that in the Alger study, both immunization and challenge doses were administered intraperitoneally, which may result in a significant degree of nonspecific protection (Stauber 1963). Further, as pointed out by Spitalny and Nussenzweig (1973), mosquitoes transmit malarial parasites by intravenous inoculation of sporozoites and not intraperitoneally. It would appear that this approach will only be significant if it can be shown to protect against challenge by the intravenous route.

While our results do not permit us to conclude that higher levels of mosquito antigen would not produce a level of immunity comparable to that observed by Alger and her co-workers, the data do permit the conclusions that mosquito antigens present in inocula of irradiated sporozoites used in the immunization schedules are not in themselves responsible for the protective immunity observed.

One major practical consideration yet to be resolved centers on obtaining an adequate source of the antigen (Ward and Hayes 1972; Beaudoin *et al.* 1974a). This certainly is one of the key points which must be addressed as development of a malaria vaccine begins in earnest.

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